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RESEARCH ARTICLE

Effect of *hxk2* deletion and *HAP4* overexpression on fermentative capacity in *Saccharomyces cerevisiae*

Jasper Merijn Schuurmans[1], Sergio L. Rossell[2], Arjen van Tuijl[2], Barbara M. Bakker[2], Klaas J. Hellingwerf[1] & Maarten Joost Teixeira de Mattos[1]

[1]Department of Molecular Microbial Physiology, Swammerdam Institute of Life Sciences, University of Amsterdam, Nieuwe Achtergracht, Amsterdam, The Netherlands; and [2]Department of Molecular Cell Physiology, Vrije Universtiteit, De Boelelaan, Amsterdam, The Netherlands

Correspondence: M. Joost Teixeira de Mattos, Department of Molecular Microbial Physiology, Swammerdam Institute of Life Sciences, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands. Tel.: +31 (0) 205257066; fax: +31 (0) 205257056; e-mail: teixeira@science.uva.nl

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Abstract

To describe the fermentative potential of a yeast cell, the fermentative capacity (FC) has been defined as the specific rate of ethanol and CO_2 production under anaerobic conditions. The effect of growth rate on FC of glucose-limited grown *Saccharomyces cerevisiae* strains with altered expression of two major glycolytic regulators, Hap4p and Hxk2p, was compared with their parent strain. Whereas overproduction of Hap4p behaved similar to the wild-type strain, deletion of *hxk2* resulted in a very different FC profile. Most importantly, with maltose as the carbon and energy source, the latter strain expressed an FC twofold that of the wild type. Further analysis at the level of gene expression showed large changes in *ADH2* transcripts and to a lesser extent in hexose transporters and genes involved in the glyoxylate cycle. With respect to primary glucose metabolism, a shift in the type of hexose transport to one with high affinity was induced. In accordance with the phenotype of the mutant strain, the maltose transporter was constitutively expressed under glucose-limited conditions and synthesis increased in the presence of maltose.

Introduction

The yeast *Saccharomyces cerevisiae* has been used for centuries to make several foods and beverages, such as bread, beer and wine. Although this yeast is one of the best-studied organisms today, many aspects of the mechanisms of fermentation are not yet well understood. The presence of oxygen, the availability of glucose and many other physico-chemical parameters of the environment play a role in the net distribution of the carbon fluxes that conserve energy, i.e. fermentation and respiration. In industrial application, control of this distribution is essential and a fermentative flux may be desired when CO_2 and/or ethanol are the preferred end products: this is the case e.g. during the raising of dough where the yeast should ferment sugars to CO_2 with minimal increase in biomass. To quantify the potential to produce CO_2 and ethanol, the rate at which this process occurs is defined as the fermentative capacity (FC) of the particular yeast strain used. Maximalization of the FC is important, for example because in the late industrial production phase *S. cerevisiae* tends to grow very slowly due to oxygen- and heat-transfer problems in the fermentors. Slower growing cells express a lower FC (Van Hoek *et al.*, 1998) and attempts to increase their performance by overexpression of the lower part of glycolysis have been only partly successful (Smits *et al.*, 2000). Another problem during the production of yeast is the tendency of this organism to ferment even in the presence of excess oxygen. This so-called Crabtree effect (Van Urk *et al.*, 1989) affects the biomass yield dramatically as a consequence of the low energy efficiency of fermentation. Altering the metabolic network in key places could shift metabolism to a greater respiratory output, thus increasing the biomass yield. An important role in controlling the catabolic flux distribution over respiration and fermentation has been ascribed to the global glycolytic regulators Hap4p and Hxk2p (Blom *et al.*, 2000; Diderich *et al.*, 2001b; van Maris *et al.*, 2001). In this study, it was sought out to investigate the effects on FC of a hexokinase II deletion strain (*hxk2Δ*) and the hem-activated protein 4 overexpression strain (*HAP4*↑). Both strains show an increased respiratory output under glucose-excess conditions (Blom *et al.*, 2000), suggesting that their ability to

ferment under anaerobic conditions could be impaired. The effects were studied when cells were grown on glucose, which is baker's yeast's preferred carbon source as well as on maltose, which is the most abundant sugar in bread dough. The observed differences between the *hxk2Δ* and the wild-type strain with respect to FC and physiological behavior are complemented by an analysis at the level of the transcriptome, of the glycolytic proteome including the sugar transport proteome.

Materials and methods

Strains and growth conditions

The strains used were CEN.PK113-7D (MATa URA3 LEU2 HIS3 TRP1 SUC2 MAL MEL GAL) provided by Dr P. Koetter (Frankfurt, Germany), an *HAP4* overexpression strain [described previously (van Maris *et al.*, 2001), a *hxk2*Δ:KanMX4 mutant (described previously (Diderich *et al.*, 2001a)]. These strains were grown in laboratory fermenters (L.H. Engineering, Maidenhead, UK) under aerobic glucose-limited conditions (Van Hoek *et al.*, 1998). A defined mineral medium described by Verduyn *et al.* (1992) was used. The concentration of glucose in the feed was $5\,g\,L^{-1}$. The working volumes used varied between 0.65 and 0.8 L. pH was set at 5.0 ± 0.1 by automatic addition of 1 M NaOH. BDH Laboratory Supplies Silicone antifoaming agent was used in a $50\,\mu L\,L^{-1}$ concentration to prevent foam development. The cultures were sparged with air at a flow rate of *c.* 1 volume of air per vessel volume per minute. The cultures were stirred with double impellers (LH Engineering) at an equal r.p.m. as the culture volume. The cultures were grown in the continuous mode by steady addition of medium while the overflow medium was disposed as waste. After 6–7 volume throughputs, a steady state was considered to be reached as biomass and carbon dioxide (CO_2) and O_2 did not vary more than 5% after this time. Oxygen consumption and CO_2 production were determined by passing the effluent gas through an paramagnetic oxygen analyzer type 1100 (Servomex, Crowborough, UK) and an IR gas analyzer type 4100 (Servomex, Crowborough, UK), respectively.

Sample extraction and analysis

Protein concentrations were determined according to the method of Lowry *et al.* using fatty-acid-free bovine serum albumin as a standard. Samples taken for metabolite analysis were immediately treated with 35% cold (4 °C) perchloric acid, and stored at −20 °C. Upon analysis, samples were neutralized with 7 M KOH and filtered through a 0.45-μm filter. Dry weight was determined by placing 2×10 mL of culture in preweighed tubes; the culture was then spun down at 3000 ***g*** (Sorvall, SS-34 rotor) at 4 °C, then washed with demineralized water, spun down again and dried overnight at 100 °C. The difference in weight was used to determine the dry weight of the culture.

Metabolites were determined by means of HPLC (LKB, Bromma, Sweden) with a Rezex organic acid analysis column with an 8-μm particle size, 8% cross-linking and a hydrogen ionic form (Phenomenex, Torrance, CA) at a temperature of 45 °C and with 7.2 mM H_2SO_4 as an eluent. Detection was performed with an RI1530 refractive index detector (Jasco, Tokyo, Japan). Peak integration and data processing were performed with BORWIN CHROMATOGRAPHY software (Le Fontanil, France).

FC assays

The method used was essentially as described by Van Hoek *et al.* (1998). Samples containing 100 mg of dry weight of biomass from a steady-state culture were harvested by centrifugation at 3000 ***g*** (Sorvall, SS-34 rotor) for 10 min, washed once with defined mineral medium (Verduyn, 1992) containing no carbon source and resuspended in 10 mL of a $5\times$ concentrated medium again lacking a carbon source. These cell suspensions were then introduced into a thermostatted (30 °C) vessel; the volume was adjusted to 45 mL with demineralized water. After 10 min of incubation, 5 mL of a 20% glucose (w/v) or maltose solution was added. Samples were taken at appropriate time intervals. The headspace was continuously flushed with water-saturated N_2 gas at a flow rate of *c.* $50\,mL\,min^{-1}$. Metabolite concentrations were determined using the HPLC. FC was calculated from the increase in ethanol concentration over 30 min for glucose and over 60 min for maltose. The increase in biomass was negligible during the assay.

Preparation of cell extracts

Culture samples were harvested by centrifugation, washed twice with 10 mM potassium-phosphate buffer, pH 7.5, containing 2 mM of EDTA, concentrated fourfold and stored at −20 °C. Before use, the samples were thawed, washed and resuspended in 100 mM potassium-phosphate buffer, pH 7.5, containing 2 mM $MgCl_2$ and 1 mM dithiothreitol. Extracts were prepared by sonication with 0.7 mm-diameter glass beads at 0 °C in an MSE sonicator (Abcoude, Netherlands) (150 W output, 7 μm peak-to-peak amplitude) for 3 min with 0.5-min intervals of cooling. Unbroken cells were removed by centrifugation at 4 °C for 20 min at 12 000 ***g*** (Sorvall SS-34). The supernatant was used as the cell extract.

Preparation of total RNA and labeled cRNA

Samples for RNA isolation were collected each hour from a batch fermentor's culture at appropriate time points,

flash-frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted using the method described in Zakrzewska *et al.* (2005). The concentration and quality of RNA were determined by measuring $A_{260\,nm}$, $A_{280\,nm}$ and $A_{230\,nm}$ on a Nanodrop spectrophotometer. The purity and integrity of the RNA samples were further validated with RNA LabChip on a 2100 Bioanalyzer from Agilent Technologies.

Total RNA was labeled according to the manufacturer's protocol (Affymetrix). Total RNA (20 μg) was used for first-strand cDNA synthesis. This was followed by synthesis of second-strand cDNA. cDNA was purified using the GeneChip Sample CleanUp Module from Qiagen. The cDNA was used for the synthesis of biotin-labeled cRNA, which was performed with the ENZO BioArray HighYield RNA Transcript Labeling Kit from Affymetrix. The synthesized cRNA was purified with the GeneChip Sample CleanUp Module from Qiagen. The concentration and quality of labeled cRNA were tested using a spectrophotometer. Subsequently, the cRNA fragmentation reaction was carried out according to the manufacturer's protocol. The degree of fragmentation was confirmed with RNA LabChip. The samples were stored at −20 °C before hybridization.

Hybridization and scanning of the DNA microarrays

The biotin-labeled cRNA samples were hybridized to the Affymetrix GeneChip® Yeast Genome S98 Array according to Affymetrix protocols (http://www.affymetrix.com). This chip contains 25-mer oligonucleotide probes for *c.* 6400 *S. cerevisiae* ORFs. Each ORF is represented by *c.* 16 probes, covering different parts of its sequence. Every probe is neighbored by a probe that is identical, except for one nucleotide in the middle of its sequence. This probe is called the 'mismatch' probe (MM), as opposed to the 'perfect match' probe (PM). The arrays were scanned with the GeneArray Scanner System on standard settings at 3 mm resolution. The data were extracted from the scanned images with MAS 5.0 (Microarray Suite 5.0).

Data processing

DCHIP was used to normalize the raw data. DCHIP is a software package implementing model-based expression analysis of oligonucleotide arrays and several high-level analysis procedures (Li & Hung Wong, 2001). The model-based approach allows probe-level analysis on multiple arrays. The arrays were normalized by adjusting the overall brightness of the arrays to a similar level. Background subtraction was performed before calculating the expression values. Expression values were calculated using the perfect match model only, as this is unaffected by the adverse effects of mismatch probes. A more detailed description of the procedure used is given in (Zakrzewska *et al.*, 2005).

Enzyme determinations

Enzyme assays were performed in a COBAS BIO automatic analyzer (Roche Diagnostics, Mannheim, Germany) at 30 °C, measuring at 340 nm (E340 nm of reduced pyridine-dinucleotide cofactors = 6.3 mM^{-1}) with freshly prepared cell extracts. All enzyme activities are expressed as μmol min^{-1} (mg protein)$^{-1}$. When necessary, samples were diluted with demi-water. All assays were performed with three concentrations of cell extract to confirm that the reaction rates were proportional to the amount of cell extract added. All enzymes were assayed according to van Hoek *et al.* (1998).

Zero *trans*-influx assays

Zero *trans*-influx rates of sugars were determined in a 5-s assay according to Walsh *et al.* (1994) at 30 °C in the same medium that was used for growth (pH = 5.0). Maltose experiments were performed in the same way using ^{14}C-labeled maltose. For the maltose experiments, the transport time was increased from 5 to 15 s. Kinetic parameters of sugar transport were derived from least square fitting of the data to one- or two- component Michaelis–Menten models using SIGMAPLOT software.

Results and discussion

The CEN.PK113-7D (wild-type), *hxk2*Δ and *HAP4*↑ strains were grown in glucose-limited aerobic chemostats at three different specific growth rates, 0.1 h^{-1} (slow growth), 0.31 h^{-1} (the fastest growth rate with fuly respiratory catabolism) and slightly higher (0.32 h^{-1}), where steady-state catabolism starts to become fermentative for the wild type i.e. just above its critical specific growth rate. For all data presented here, carbon balances all fitted within the 90–110% range. At low growth rates, all strains were fully respiratory and thus did not form ethanol. Fluxes and yields are equal under these conditions, indicating than their energetic demands are the same. This appears to be true up to a specific growth rate of 0.31 h^{-1}. The critical dilution rate (D_c) for the wild type and *HAP4*↑ turned out to be 0.32 h^{-1}: both strains catabolize glucose at this growth rate respiro-fermentatively with a concomitant decline in $Y_{glucose}$ of 20%. The *hxk2*Δ strain, however, continued to grow fully respiratory and as a consequence maintained its biomass yield value on glucose (see Table 1). The higher respiratory capacity in the *hxk2*Δ strain has been observed under other growth conditions as well and has been subject to a number of studies (Petit *et al.*, 2000; Diderich *et al.*, 2001a; Raghevendran *et al.*, 2004). From the steady-state chemostat cultures described above, samples for FC analysis were taken. From Fig. 1, it can be seen that the FC on glucose ($FC_{glucose}$) for both the wild-type and the *HAP4*↑ strain

Table 1. Physiology of the wild-type, *hxk2Δ* and *HAP4*↑ strains

D (h^{-1})	$q_{glucose}$ (mmol g^{-1} h^{-1})	$q_{ethanol}$ (mmol g^{-1} h^{-1})	qO_2 (mmol g^{-1} h^{-1})	qCO_2 (mmol g^{-1} h^{-1})	$Y_{glucose}$ (g/g)	C%
WT						
0.1	1.1	0	2.6	2.8	0.51	106
0.31	3.4	0	8.3	8.8	0.52	106
0.32	4.4	7.1	7.1	9.5	0.41	102
*hxk2*Δ						
0.1	1.1	0	2.2	2.5	0.52	100
0.31	3.2	0	6.2	6.2	0.54	97
0.32	3.4	0	8.5	8.9	0.53	107
HAP4 ↑						
0.1	1.1	0	3.0	3.0	0.52	110
0.31	3.1	0	7.6	7.8	0.57	108
0.32	4.3	2.7	7.0	9.7	0.42	110

Strains were grown in glucose-limited aerobic chemostats at various dilution rates. Fluxes were determined for glucose, ethanol, CO_2 and O_2. The biomass yield on glucose and the carbon balance are also shown.

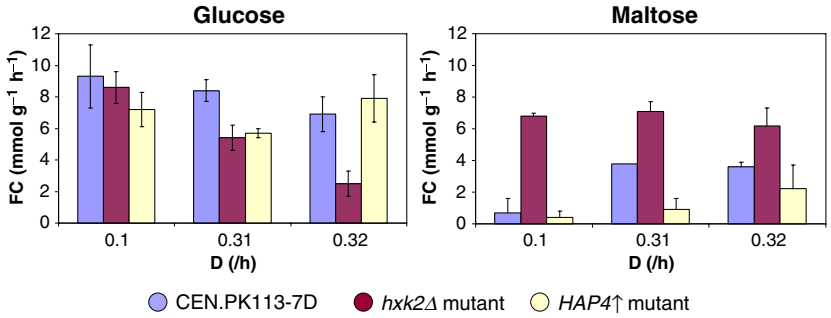

Fig. 1. FC of the wild-type, *hxk2Δ* and *HAP4*↑ strains. Left: $FC_{glucose}$ of the wild-type (blue) *hxk2Δ* (red) and *HAP4*↑ (yellow) strains. Right: $FC_{maltose}$ of the wild-type (blue) *hxk2Δ* (red) and *HAP4*↑ (yellow) strains. The results of at least two independent experiments are shown, and the SD is shown in error bars.

hardly changed with changing growth rates, whereas the $FC_{glucose}$ of the *hxk2*Δ strain declined dramatically with increasing growth rates, resulting in a 75% reduction of capacity at the highest growth rate tested. Even more remarkable is the observation that deletion of *hxk2* resulted in a strikingly different behavior when maltose was used as the carbon and energy source. Neither the wild type nor the *HAP4*↑ strain expressed a significant FC on maltose ($FC_{maltose}$) upon transfer from glucose-limited growth conditions to the FC assay with maltose whereas the *hxk2*Δ strain was able to catabolize this sugar immediately at a rate that was some 70% of the $FC_{glucose}$ of the wild type. In addition, the $FC_{maltose}$ did not change with the growth rate. In contrast, the behavior on maltose of the *HAP4*↑ strain was opposite to that of the *hxk2*Δ strain (Fig. 1) in that it expressed a lower $FC_{maltose}$ than the wild type.

Further comparative studies were performed with the wild-type and *hxk2*Δ strains, at a dilution rate of 0.32 h^{-1}, because here the largest physiological differences were observed. Transcriptome profiles as well as the activities of putatively important enzymes such as those of glycolysis and the sugar transporters were analysed. The analyses were carried out for three points in time, namely before $FC_{maltose}$ incubation, after 30 min and after 60 min of $FC_{maltose}$ incubation. To express the differences in $FC_{glucose}$, to the samples before incubation on maltose are referred.

In Fig. 2, a comparison of all transcripts in the central carbon metabolism of yeast is shown. In accordance with the similar behavior of the wild type and the *HAP4*↑ strain under the conditions tested, the transcriptome profiles of these strains do not show major differences, with the exception of downregulation of the *HXT4* transcript and some upregulation for *ICL1* and *PCK1* transcripts in the *HAP4*↑ strain.

Also in accordance with major physiological changes due to deletion of *hxk2*, the *hxk2*Δ strain shows several more distinct differences at the expression level. As expected, the *HXK2* transcript is hardly present in the mutant. Further, the expression of *ADH2* had more than doubled as has been observed earlier for batch cultures with excess glucose (Schuurmans *et al.*, 2008) Also, several genes involved in the glyoxylate cycle, the tricarboxylic acid cycle and *FBP1*

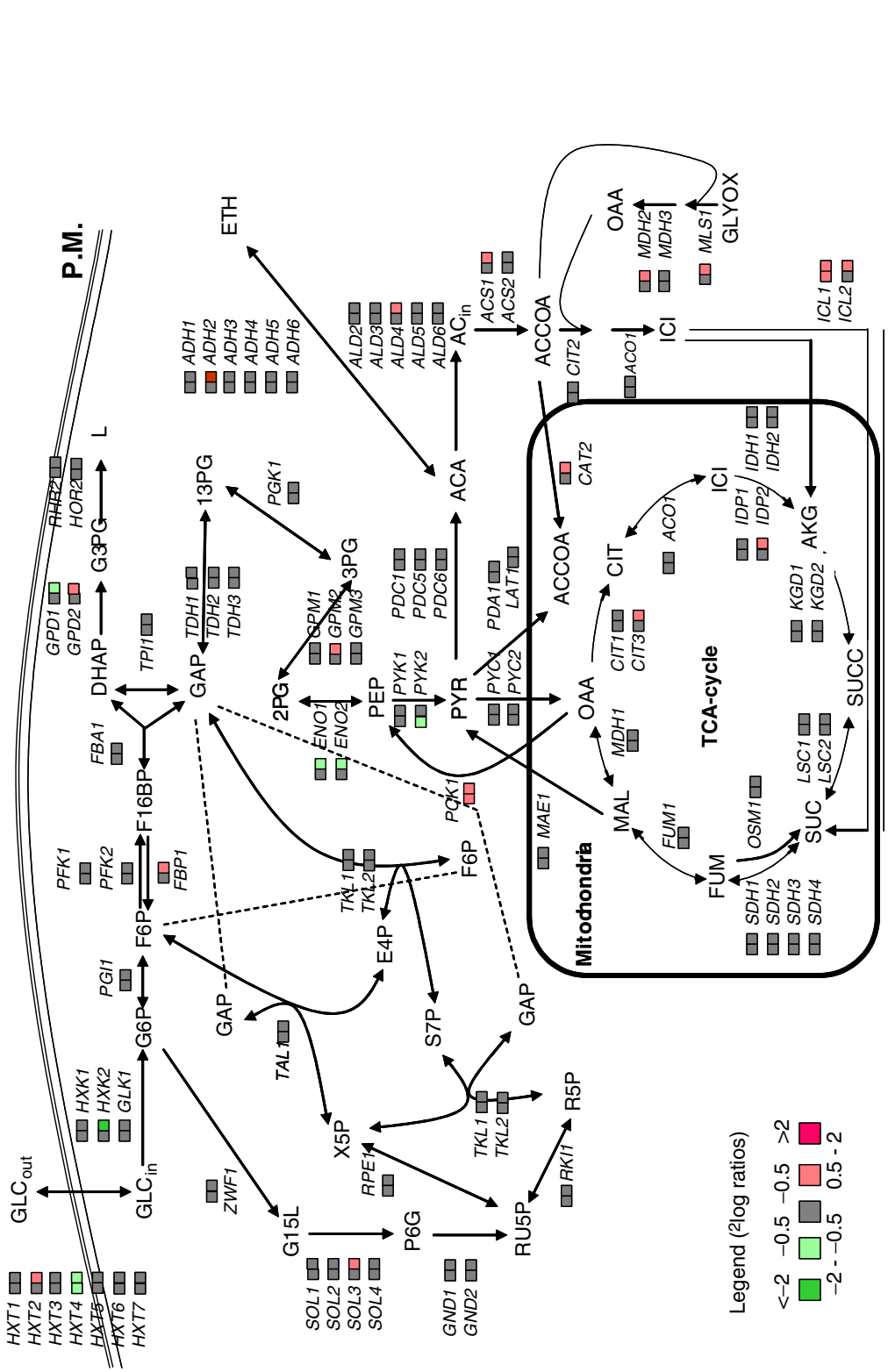

Fig. 2. Gene expression ^{2}log-ratios in the central carbon metabolism of yeast. Transcriptome data from the mutants and the wild type, taken during steady-state growth at $D = 0.32\ h^{-1}$, were normalized and expression ratios were calculated relative to the wild type. The expression ratios of most of the genes involved in the depicted pathways are shown here. The values are expressed as ^{2}log-ratios and are relative to the wild type. The left squares show the *HAP4*↑/WT ratio and the right squares show the *hxk2*Δ/WT ratio.

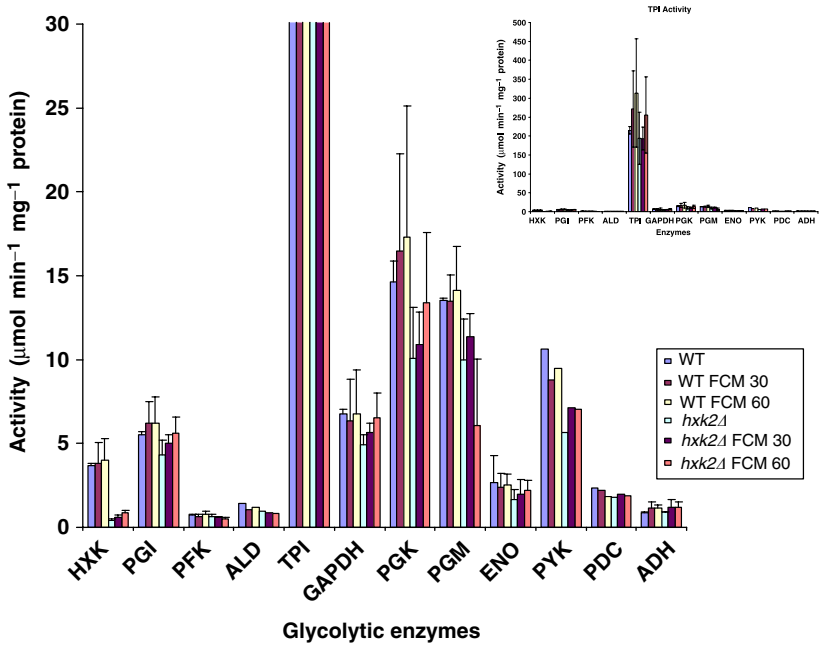

Fig. 3. Glycolytic enzyme activities in the wild type and *hxk2Δ* strain, before, during and after FC incubation on maltose. The activities of the wild-type strain before (blue), during (red) and after (yellow) incubation with maltose are shown. Activities of the *hxk2*Δ strain are shown in cyan (before maltose incubation), purple (during maltose incubation) and orange (after maltose incubation).

(needed for gluconeogenesis) are increased. Although these results point towards gluconeogenic growth in the *hxk2*Δ strain, from alcohol as a substrate, this cannot explain the difference in $FC_{glucose}$, as gluconeogenesis in yeast requires oxygen.

Further information at the proteome level of glycolysis is presented in Fig. 3 where glycolytic enzyme activities are presented. These show the V_{max} of each of the glycolytic enzymes and thus represent the maximum sustainable flux per enzyme (and not the total *in situ* flux, as shown in Table 1). Assuming that cells contain *c.* 40% protein, the values can be recalculated to allow direct comparison with the measured fluxes of glucose and ethanol. Thus, triose phosphate isomerase (TPI) activities are much higher than any of the other glycolytic enzymes, in the range of 25–45 μmol min mg^{-1} DW in contrast to a 0.1–3 μmol min mg^{-1} DW for all the other enzymes for both yeast strains. Relatively low activities were found for phosphofructokinase, aldolase and alcohol dehydrogenase (ADH) but according to the above calculation, the sustainable flux through these enzymes still greatly exceeds the *in situ* flux, e.g for phosphofructokinase (wild type before FC incubation), the V_{max} is 0.22 μmol min^{-1} mg^{-1} DW, which corresponds to 13.2 mmol h^{-1} g^{-1} DW, whereas the *in situ* flux is 4.4 mmol h^{-1} g^{-1} DW. Hexokinase (HXK) activity is much lower in the *hxk2*Δ strain; therefore, it can be concluded that the lack of one of its three hexokinase iso-enzymes is not compensated by enhanced expression of either or both of the other two. Again, the maximum activity greatly exceeds the *in vivo* flux, (7.8 mmol h^{-1} g^{-1} DW vs. 3.4 mmol h^{-1} g^{-1} DW, respectively). Changes in enzyme activity were also observed during the $FC_{maltose}$: wild-type enzyme activity appears to increase after 30 min, but in the end is lower in all cases than the starting activity, whereas enzyme activity in the *hxk2*Δ strain increases in 30 min, but does not decline like in the wild type after an hour. An adaptation to maltose occurs during the incubation, but the observed changes in $FC_{maltose}$ in time cannot be explained by changes in glycolytic enzyme activities. Likewise, the poor performance of the *hxk2*Δ strain on glucose is not entirely clear, although the reduction in hexokinase activity in this strain can account for at least a 40% reduction in $FC_{glucose}$. This still leaves 35% of the reduction in $FC_{glucose}$ unexplained.

To further investigate the changes in $FC_{glucose}$, glucose transport kinetics were studied by zero *trans*-influx transport assays. These results are presented in Fig. 4. The wild-type strain, when sampled from glucose-limited chemostat cultures ($D = 0.32\ h^{-1}$) showed a one-component transport system, similar to that found in earlier studies (Diderich *et al.*, 1999). The V_{max} found in this study is slightly lower; however, the experiments were performed in unbuffered medium at pH 5.0, whereas the earlier experiments were performed in phosphate buffer at pH 6.5. The overall transport is characterized as a high-affinity system ($K_{m,\ glucose} = 1.2$ mM), which is most likely solely attributable to Hxt6p/Hxt7p. In contrast, the *hxk2*Δ strain shows a two-component system, with a $K_{m,\ glucose}$ of 14.1 mM, suggesting the significant contribution of the Hxt2-transporter and a very high affinity component with a K_m of only 0.2 mM. This very high-affinity component has been observed before (J.A. Diderich & M.C. Walsh, unpublished data), but it is unknown which hexose transporter manifests this very high

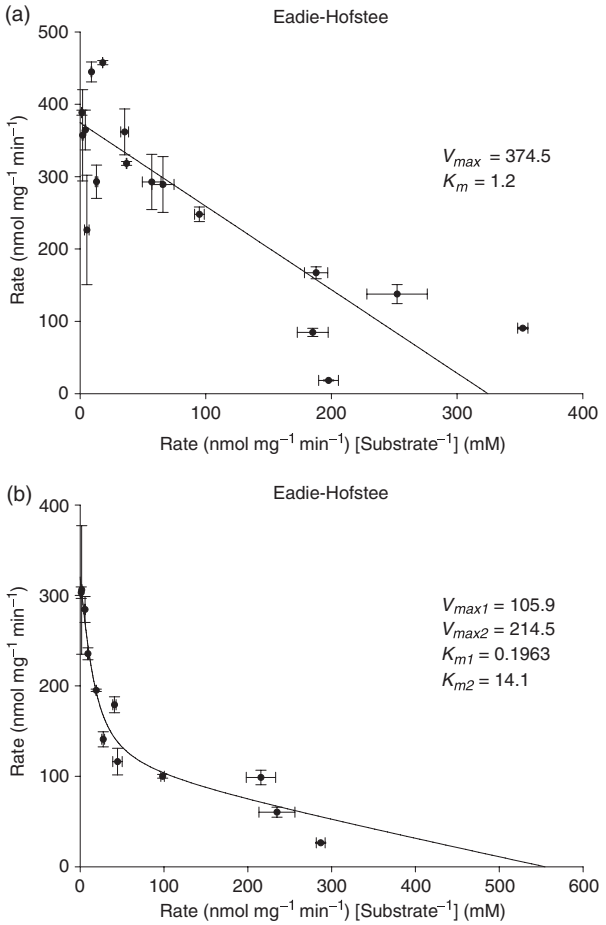

Fig. 4. Eadie–Hofstee plots of the glucose transport kinetics of the wild type (a) and *hxk2*Δ strain (b). Samples were obtained from glucose-limited aerobic cultures at a dilution rate of 0.32 h^{-1}. Velocities are shown in nmol min^{-1} mg protein^{-1}, the affinity constants are shown in millimolar. SE *hxk2*ΔV_{max1} 35.9, V_{max2} 32.5, K_{m1} 0.19, K_{m2} 8.2. SE wild-type V_{max} 14.0, K_m 0.24.

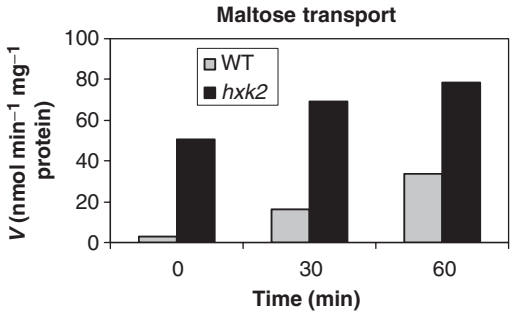

Fig. 5. Rate of maltose transport by the wild type and the *hxk2Δ* strains. Samples were obtained before, during and after FCM. The cells originated from glucose-limited aerobic cultures at a dilution rate of 0.32 h^{-1}. Velocities are shown in nmol min^{-1} mg protein^{-1}.

affinity. FC on glucose is measured in an excess glucose environment and because the total V_{max} for glucose transport of both strains does not vary considerably, the different values for the $FC_{glucose}$ cannot be explained by a change in transport capacity.

It was tested whether the capacity to ferment maltose was related to the presence of a maltose transport system. Indeed, it was found (Fig. 5) that maltose transporters are functional in the *hxk2*Δ strain during growth on glucose whereas this was not the case for the wild type. The latter strain expressed transport activity only after an induction period in the presence of maltose and reached after *c.* 1 h an activity that equaled the activity of the *hxk2*Δ strain from the start of maltose incubation. These differences can account for the exceptional performance of the *hxk2*Δ strain compared with the wild type, as it can start fermenting maltose immediately, plus more maltose transporters are induced, just like in the wild type, by the presence of maltose.

Previously, it has been shown that both the *hxk2*Δ and *HAP4*↑ strains in batch cultures express a more efficient glucose catabolism due to an increase in respiratory capacity and altered regulation of carbon source utilization, resulting in higher biomass yield values (Schuurmans *et al.*, 2008). Increased yield values may have an economic advantage in the production process but because under aerobiosis these mutants rely less on their fermentative branch of glycolysis, the question then arises as to the capacity of these strains to ferment sugars when transferred to anaerobic conditions (as e.g. occurs when yeast is applied in dough). It was therefore sought to quantify the trade-off between better growth performance and FC. The results of this study show the complexity of the regulatory network of yeast glycolysis: loss of hxkp results in a decreased capacity to ferment glucose but maltose can be fermented immediately, a property that is not observed in wild-type cells. Overproduction of Hap4p, on the other hand, has no effect on the capacity to ferment glucose but does not endow the cells to cope directly with maltose. The question then arises as to whether these physiological responses can be explained at the level of the transcriptome in general or at the level of specific enzyme activities. Although a lower hexokinase activity in the *hxk2*Δ strain was observed, mostly due to the deletion of one of the hexokinase iso-enzymes the alternative phosphorylating enzymes, *GLK1* and *HXK1*, can still account for enough activity to sustain a higher glucose influx than observed *in vivo*. This is true for all the other enzyme activities in the glycolytic path. Interestingly, TPI activity was much higher than the other glycolytic enzymes. This has been observed by others (Rossell *et al.*, 2008), and could possibly be seen as a mechanism to warrant fast conversion between di-hidroxyacetone phosphate and glyceraldehyde-3-phosphate in order to maintain redox neutrality. When the *hxk2*Δ strain was transferred to fermentative conditions

with maltose, glycolytic enzyme activity was induced, where the wild-type activity was decreased after an hour. It has been shown (Gancedo, 1998) that the *hxk2*Δ strain lacks carbon catabolite repression. This is in accordance with the observations that both maltose transporter and maltase activity may already be present during growth on glucose, whereas in the wild type it is still actively repressed. Thus, it may be that in contrast to the wild-type cells, the *hxk2*Δ strain produces a low level of glucose upon transfer to a maltose-containing environment by the activity of maltase, which subsequently diffuses out of the cells through the glucose transporters as was reported earlier (Jansen *et al.*, 2002). These low levels may then result in the upregulation of glycolysis.

Cells lacking Hxk2p and hence partially derepressed express a more oxidative glucose catabolism. Indeed, transfer to an anaerobic environment with glucose results in a much lower FC than observed for the wild type. However, from the results with maltose, where high ethanol production rates are found, it must be concluded that the capacity of the pathway from glucose-6-phosphate to ethanol is not diminished by the absence of Hxk2. This implies that the cause of the shift to a more oxidative physiology resides in the early steps of glucose metabolism: transport and/or the initial phosphorylation. Although a change in expression of glucose transporters does occur, it remains to be elucidated whether the catabolic shift is evoked directly by transcriptional changes or indirectly by the accompanying changes in glycolytic intermediates (and therefore by alterations in the steady-state kinetics of glycolysis). Hierarchical regulation analysis (Rossell *et al.*, 2006) could be the appropriate tool to solve this question.

Whereas it has been shown previously (Schuurmans *et al.*, 2007) that Hxk2 and HAP4 act synergistically with respect to their control on the flux distribution over oxidative and fermentative glucose catabolism, no significant role could be seen for HAP4 with respect to adaptation to fermentative maltose conditions. It is concluded therefore that the impact of Hxk2 on the physiology of *S. cerevisiae* with sugar metabolism is more profound and more related to catabolite repression and the utilization or exclusion of alternative carbon and energy sources than that of HAP4, which seems to be limited to the regulation of the two catabolic modes only.

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